Intragenomic Repair Heterogeneity of DNA Damage

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The mutagenic and carcinogenic consequences of unrepaired DNA damage depend upon its precise location with respect to the relevant genomic sites. Therefore, it is important to learn the fine structure of DNA damage, in particular, proto-oncogenes, tumor-suppressor genes, and other DNA sequences implicated in tumorigenesis. Both the introduction and the repair of many types of DNA lesions are heterogeneous with respect to chromatin structure and/or gene activity. For example, cyclobutane pyrimidine dimers are removed more efficiently from the transcribed than the nontranscribed strand of the *dhfr* gene in Chinese hamster ovary cells. In contrast, preferential strand repair of alkali-labile sites is not found at this locus. In mouse 3T3 cells, dimers are more efficiently removed from an expressed proto-oncogene than from a silent one. Persistent damage in nontranscribed domains may account for genomic instability in those regions, particularly during cell proliferation as lesions are encountered by replication forks. The preferential repair of certain lesions in the transcribed strands of active genes results in a bias toward mutagenesis owing to persistent lesions in the nontranscribed strands. Risk assessment in environmental genetic toxicology requires assays that determine effective levels of DNA damage for producing malignancy. The existence of nonrandom repair in the mammalian genome casts doubt on the reliability of overall indicators of carcinogen–DNA binding and lesion repair for such determinations. Tissue-specific and cell-specific differences in the coordinate regulation of gene expression and DNA repair may account for corresponding differences in the carcinogenic response to particular environmental agents.

Introduction

Humans and other organisms are constantly exposed to a wide variety of potentially harmful environmental chemical and physical agents, many of which damage DNA, causing mutations or deletions of vital genetic information. Among the possible consequences of these events are the initiation of cancer and cell death. Repair pathways have evolved for excising damaged nucleotides from DNA; these maintenance mechanisms assist in ameliorating the detrimental effects of genotoxic entities and appear to be indispensable for normal development.

In recent years, emphasis has been placed on examining DNA repair in defined domains of chromatin (1–4). In some instances, removal of damage has been shown to be heterogeneous within the genome, occurring at dissimilar rates and to varying extents in different DNA sequences. Although the specific regulatory parameters remain to be elucidated, there is a consistent thread of observations of phenomena that appear to be the foundation for preferential DNA repair: The removal of some types of damaged

bases from discrete sequences of the genome corresponds to the transcriptional activity in those sequences.

Nonrandom clearance of DNA damage based on gene expression indicates a re-evaluation of the existing policy of using data on carcinogen–DNA binding and lesion repair in the total genome for assessing carcinogenic and toxic risks. Tissue-specific and cell-specific differences in gene expression and its regulation may contribute to the reported variations in tumorigenic and cytotoxic responses to certain environmental and experimental agents.

Excision Repair Pathways

Two general pathways exist for excising damaged bases from DNA: nucleotide-excision repair and base-excision repair (5,6). Nucleotide-excision repair is responsible for the removal of a wide variety of bulky adducts from DNA, including lesions resulting from exposure to ultraviolet (UV) radiation, aflatoxins, furocoumarins, and aminofluorene derivatives. In both prokaryotes and eukaryotes, incision at the site of damage occurs via the formation of a multiprotein complex; however, recognition of the adducted base does not appear to be the principal factor that summons nucleotide-excision repair. The actual signal may well be conformational distortion of the double helix at the site of damage; this would explain how one system can remove a wide range of different lesions. In *Escherichia*

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coli, incision requires a complex consisting of three proteins collectively referred to as the UvrABC excinuclease (7). In human cells, this process is significantly more complicated and less well understood. On the basis of complementation studies with cells obtained from patients with the nucleotide-excision repair deficiency disease xeroderma pigmentosum, as many as seven different gene products have been implicated in the incision step. Following excision of a section of the damaged DNA strand, DNA polymerase δ synthesizes a repair patch using the undamaged strand as a template (8). Subsequently, the newly made DNA is sealed to the contiguous DNA by DNA ligase (5,6).

Lesions in DNA that do not cause major distortions of the helix are often removed by base-excision repair (5,6,9,10). Among these adducts are 7-methylguanine and 3-methyladenine. The initial step in this repair sequence involves excision of the damaged base by a glycosylase, leaving an abasic site in the DNA that is then a substrate for an endonuclease. In contrast to nucleotide-excision repair, in base-excision repair the generally small glycosylases recognize specific damage in the DNA. For example, 3-methyladenine and 7-methylguanine are removed by 3-methyladenine–DNA glycosylase. Following endonucleolytic cleavage, resynthesis of the DNA and ligation occur; however, in the case of base-excision repair, DNA polymerase β mediates the process of gap filling, and the patches are normally only one to three nucleotides long (8). There is some overlap in the recognition features of the respective pathways, such that some lesions may be effectively repaired by either scheme.

Intragenomic Repair Heterogeneity in Cells Derived from Vertebrates

Ultraviolet Radiation

The majority of adducts formed in DNA following cellular exposure to short-wavelength UV light are cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PP). CPDs pose hurdles to replication, and they block transcription, making them important contributors to both the mutagenic and lethal effects of UV radiation (11). In light of the deleterious nature of these lesions, it was puzzling to radiation biologists to find that rodent cells, in general, survive quite well following exposure to UV light, in spite of the fact that they remove CPDs very poorly from their total cellular DNA (12). The apparently incongruous nature of these observations was partially clarified by Bohr et al. (13). Using T4 endonuclease V, an enzyme that cleaves DNA specifically at CPDs, in conjunction with denaturing gel electrophoresis and Southern analysis, they demonstrated very efficient removal of dimers from the actively expressed dihydrofolate reductase (*dhfr*) gene of Chinese hamster ovary (CHO) cells, suggesting that certain discrete regions of the genome are subject to selective nucleotide-excision repair. Mellon et al. (14) then found that removal of dimers is limited to the transcribed strand, strongly hinting at a direct link between DNA repair and gene expression. These findings may well explain why rodent cells with limited nucleotide-excision repair capabilities survive so well: Perhaps they selectively remove damage from essential, active regions of the genome as a means of protection from the lethal consequences of UV irradiation and simply dispense with repair in other domains, thereby accepting the mutagenic consequences of translesion synthesis.

Studies with other rodent cells confirmed preferential removal of CPDs from active regions. Following exposure to UV radiation, the expressed c-*abl* proto-oncogene in mouse 3T3 fibroblasts is effectively cleared of CPDs, while the damage persists in the silent c-*mos* oncogene (15). These results offer further evidence in support of the notion that transcribed DNA is more efficiently repaired than quiescent domains. They also have important implications for the process of oncogene activation, where the consequences of limited repair could lead to aberrant expression of these loci or to the generation of mutant forms.

In light of the variations in CPD clearance from proto-oncogenes, recently reported data concerning preferential DNA repair in the c-*myc* locus of BALB/c mice are very intriguing (16). Two inbred strains of mice were used for these studies — one resistant and one susceptible to plasmacytoma formation. Splenic B-lymphocytes were isolated from these two strains and grown in culture, and repair of c-*myc* was studied following UV irradiation. CPDs present in the protein-encoding portion of the gene and in the 3′ flank were efficiently cleared from the transcribed strands of both strains, but no repair was observed in the nontranscribed strand in either case. Surprisingly, repair of the regulatory region and 5′ flank occurred in both strands in the plasmacytoma-resistant strain but was absent from this locus in the tumor-susceptible strain. These results have important implications for the etiology of cancer in these mice. The specific defect in repair would appear to be linked to a regulatory locus of an oncogene rather than to the actual coding region for the gene product.

Another recent report, concerning CPD repair in the c-*ras* oncogene of cultured goldfish cells, has shown that nucleotide-excision is specifically involved in preferential DNA repair (17). These particular cells deal with CPDs in two ways: through direct reversal via photoreactivation or by nucleotide-excision repair. The results indicate that the removal of CPDs by excision repair is more efficient in c-*ras* than in the genome overall, while the efficiency of photoreversal is not affected by the location of the CPDs.

Attempts have been made to restore full competency of CHO cells in nucleotide-excision repair by transfecting the cells with heterologous repair genes (18,19). Transfection with the *den*V gene, which codes for T4 endonuclease V, restores the repair of CPDs but without preferential removal from either active versus inactive regions or the transcribed versus the nontranscribed strand of the *dhfr* gene (20,21). Therefore, most of the damaged chromatin is probably accessible to the *den*V gene product. Experiments with CHO cells that express the transfected human excision repair gene ERCC1 have yielded results similar to those found for wild-type CHO cells: The expressed *dhfr*

gene was better repaired than were silent domains, but little repair was evident on the nontranscribed strand (21,22).

The involvement of gene expression in the enhanced repair of discrete genetic loci prompted investigations to determine whether varying the parameters that regulate transcription affects preferential adduct removal. Cytosine methylation of a limited population of CG islands in DNA by DNA methylases has long been associated with fluctuations in the level of gene expression. 5-Azacytidine, an inhibitor of cytosine methylation, was used to derive a hypomethylated CHO cell line. Significant increases in the removal of CPDs from the 3′ region of the *dhfr* gene and from an extragenic region located upstream were found in these cells, suggesting a possible role for DNA methylation in regulating DNA repair (23).

Preferential removal of 6-4 PPs from the *dhfr* gene has also been documented (24). Following photo-reversal of CPDs with photolyase, 6-4 PPs remaining in isolated DNA from CHO cells were quantified using the reconstituted UvrABC excinuclease as the incising enzyme system. This strategy showed that 6-4 PPs are repaired more efficiently than CPDs in both the *dhfr* gene and in a nontranscribed, downstream region. Furthermore, the *dhfr* gene is cleared of 6-4 PPs faster than is the silent, downstream locus. It is difficult to reconcile the differential removal of CPDs and 6-4 PPs from the gene with a model that directly couples excision repair to transcription. In such a model, the frequency of transcription events would seemingly be the relevant parameter for efficient removal of all helix-distorting lesions.

Normal human cells, which remove CPDs efficiently throughout the entire genome, and those derived from patients with a variety of DNA repair deficiency diseases have been used to investigate preferential removal of UV-induced damage from specific DNA sequences. Enhanced repair of CPDs in the *dhfr* gene of normal human fibroblasts was demonstrated (25). The transcribed strand of the gene is cleared of CPDs at a faster rate than the nontranscribed strand (14) in which CPD clearance is similar to that in the total cellular DNA (26). Similar results have been found for the expressed human metallothionein *(MT)* genes using an antibody that recognizes bromouracil, and which was used to isolate DNA fragments containing bromouracil in repair patches. Following this enrichment step, labeled probes were used to detect the fragment of interest. The removal of dimers from the transcribed strand of expressed *MT* genes was approximately three times faster than that in the nontranscribed strand. The two strands of inactive genes were repaired at the same rate as the total cellular DNA (27,28).

Preferential DNA repair in humans has also been examined in a variety of cells derived from patients with DNA repair deficiency diseases. The importance of an intact nucleotide-excision repair system is highlighted by the severity of one such disorder, xeroderma pigmentosum (XP). Individuals afflicted with this autosomal recessive hereditary disease are prone to sunlight-induced skin cancers, sometimes have neurological deficits, and usually die at an early age (5). In XP-A cells, no removal of CPDs is observed from total cellular DNA or from specific DNA sequences (29,30); however, in XP-C cells, an observed low level of nucleotide-excision repair is limited to certain genomic domains (31,32). For example, the active β-actin gene in these cells belongs to a set of preferentially repaired DNA sequences 30–70 kb in length, which is, coincidentally, the size of genomic loops that extend from the nuclear matrix (33). The transcribed adenosine deaminase (*ada*) and *dhfr* genes are efficiently cleared of CPDs, as are certain nearby loci, while the unexpressed 754 locus is repaired with the same poor efficiency as the remainder of the genome (34). Patients with XP-C are defective in the repair of inactive chromatin but are still capable of clearing damage from active genetic loci.

Cockayne's syndrome is a disorder characterized by developmental deficits and photosensitivity but not by an increased risk of sunlight-induced skin cancer (5). Cells from individuals with this disorder are sensitive to UV radiation, even though CPD repair is essentially normal in the genome overall. Preferential repair of damaged DNA, however, is absent in DNA associated with the nuclear matrix, a scaffold that is affiliated with transcriptionally active loops or domains of DNA (35). Cockayne's syndome cells clear CPDs very poorly from the *ada* and *dhfr* genes early on (36), as well as from the expressed *c-abl* proto-oncogene (I. Mellon, personal communication). These data are consistent with the notion that Cockayne's syndrome involves a defect in preferential removal of damage from actively expressed regions of DNA while retaining normal repair efficiency in quiescent regions. They also support the idea that specific factors, or perhaps a distinct repair mechanism, are involved in the repair of transcribed DNA and that this particular process is somehow compromised in this disorder.

Bulky Chemical Adducts

Many chemical agents react with DNA directly or after metabolic activation to reactive species to form bulky adducts that severely distort the normal helical structure of DNA. The sequence-dependent clearance of damage due to several of these agents has been investigated as described below.

Psoralens are found in certain foods, such as celery and limes. They are bifunctional and upon photoactivation by near UV light can react with pyrimidines in DNA to form either monoadducts or interstrand crosslinks (5). Removal of psoralen–DNA lesions is deficient in the highly repetitive α DNA sequences of African green monkey cells when compared to total cellular DNA (37). In human cells, crosslinks are repaired to an extent of 80–90% in the *dhfr* gene within 24 hr but are removed very inefficiently from the silent *fms* gene. Overall repair of these cross-linking adducts in bulk DNA is, however, only 30% (38). Monoadducts are removed from the *dhfr* gene more slowly than crosslinks, being cleared to a level of 45% within 24 hr (39). Recent studies indicate that mono-adduct repair does not show strong preferential removal from active sequences (F. Baker and A. Islas, personal communication).

Aflatoxins are extremely potent liver carcinogens found in food contaminated by certain fungi. Aflatoxin B_1 can be metabolized to an agent that produces bulky DNA adducts by reaction with guanine (5). Initial damage levels in unexpressed α DNA of monkey cells are comparable to those in the genome overall, indicating homogeneous damage to the chromatin, but the damage is removed less efficiently from this locus when compared to total cellular DNA (40). Clearance of aflatoxin B_1 adducts from the transcribed strand of basally active *MT* genes of human cells is significantly faster than that of total DNA, and this repair is enhanced further by inducing these genes with $CdCl_2$ or dexamethasone. Furthermore, aflatoxin B_1 repair is deficient in a nontranscribed *MT* gene and in a processed pseudogene. After induction of transcription, the rate of adduct clearance becomes even faster in the transcribed strand, but no difference from the basal repair rate is observed in the nontranscribed strand (27). Of particular interest is the finding that α-amanatin, an inhibitor of RNA polymerase II transcription, abolishes the strand-selective repair (27), a result also seen in several other laboratories (F. Christians and P. C. Hanawalt, unpublished observations; D. Hunting, personal communication). Collectively, these results also strongly support the notion that nucleotide-excision repair is targeted to expressed regions of the genome.

Aminofluorene adduction to guanine in CHO cells treated with *N*-acetoxy-2-acetylaminofluorene is an example of a lesion that does not appear to be removed selectively from active regions of DNA (41). This finding emphasizes the need to consider the biological consequences of the adduct in question. If aminofluorene lesions in DNA were shown to have little effect on gene expression, selective repair mechanisms targeted to active genes might not be essential for reducing the harmful effects of these chemical agents.

Alkylation Damage

Alkylating agents present an important class of carcinogens and are found in food, cigarette smoke, beverages, and industrial work places (42). Some of them methylate DNA, forming a variety of adducts, including 7-methylguanine, 3-methyladenine, O^6-methylguanine, and methylphosphotriesters (43). Studies of *N*-methylpurine formation and repair in discrete segments of the genome have yielded variable results.

In those experiments in which alkylation was caused by exposure to S_N2 methylating agents, which react primarily with nitrogen and produce mostly 7-methylguanine and 3-methyladenine, little or no difference was found for the clearance of alkali-labile sites from active or inactive regions of chromatin. Alkali-labile sites formed by removal of *N*-methylpurines were not repaired preferentially in the transcribed strand of the CHO *dhfr* gene following exposure to dimethyl sulfate (44,45). Likewise, no difference in repair rates was found among the expressed *dhfr* and hypoxanthine phosphoribosyltransferase (*hprt*) genes and the silent Duchenne muscular dystrophy gene of cultured T-lymphocytes exposed to methyl methanesulfonate (46,47).

The results were frequently different and more variable when S_N1 methylating agents were used. These compounds react to a notable extent with oxygen atoms, thus producing significant levels of O^6-methylguanine and alkali-labile methylphosphotriesters, in addition to 7-methylguanine and 3-methyladenine (43). When rats were injected with *N*-nitrosodimethylamine, higher initial levels of damage were found in the expressed albumin gene than in the silent IgE gene from liver cells (48). In contrast, no difference was seen in the formation or repair of alkali-labile sites formed by the removal of *N*-methylpurines from the active collagen I gene and the inactive β globin gene of human fibroblasts following exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (49). When CHO cells were exposed to *N*-methyl-*N*-nitrosourea, the *dhfr* gene was cleared of approximately 60% of the alkali-labile sites within 24 hr, while no repair was detected in the c-*fos* gene. Overall removal of 7-methylguanine, the predominant adduct, was reported to be approximately 70% (50). While these studies support the notion that transcription is not the only factor that governs selective repair of alkali-labile sites, evidence from other experiments sustains the idea that removal of *N*-methylpurines from discrete domains of DNA is associated with the transcriptional state of the locus being investigated. Removal of alkali-labile sites formed after exposing cultured rat cells to *N*-methyl-*N*-nitrosourea was significantly more efficient from the actively expressed insulin gene in RINr 38 cells than from the unexpressed insulin gene in RINr B2 cells. Furthermore, the initial lesion frequencies in the insulin gene from both cell lines were the same (51).

These results clearly offer support for the existence of interorgan and/or interspecies differences in gene-specific repair of *N*-methylpurines; however, it is difficult to rationalize the differences in repair rates of identical adducts on the basis of the source of the methylating species used to produce them. It must be emphasized that the S_N1 methylating agents form methylphosphotriesters as approximately 12% of the total damage. These adducts are alkali-labile and do not seem to be repaired when analyzed as part of the total cellular DNA (43). If they are also not cleared from discrete genetic domains, their presence in a gene could interfere with analytical techniques involving the use of alkaline conditions. DNA would be cleaved at methylphosphotriesters, and the resulting breaks would be detected as poor repair. In order to circumvent these difficulties, it is critical to be cognizant of the number and kinds of adducts formed in the locus of interest and to consider alternative protocols in which alkaline conditions are avoided.

Intragenomic Repair Heterogeneity in Lower Eukaryotes and Bacteria

The finding that nucleotide-excision repair occurs preferentially in active genes is not limited to vertebrates. A similar phenomenon has been reported in the yeast *Saccharomyces cerevisiae* (52,53). Removal of CPDs from the transcribed strand of an active gene in a yeast minichromosome is more than five times faster than in the

nontranscribed strand, while a region downstream from the gene is cleared of CPDs at a very slow rate (*53*).

One of the strongest pieces of evidence for an effect of transcription on removal of CPDs from discrete genetic loci was obtained in *E. coli* (*54*). In the un-induced state, 50% removal of CPDs from both strands of the *lac*Z gene required 20 min. Following induction, clearance of CPDs from the transcribed strand increased in rate by one order of magnitude, while the rate of repair in the non-transcribed strand was identical to that found in the uninduced state. The implications of these results are vast. They clearly demonstrate that enhanced repair of an active locus is directly related to the level of gene expression; they show that preferential removal of damage from active genetic loci is not solely related to chromatin accessibility; and, in conjunction with the results obtained in yeast, they imply that enhanced repair of active genes has been conserved throughout evolution.

It had been hypothesized by Mellon and Hanawalt (*54*) that a blocked transcription complex may serve as an antenna for repair complexes and thus be the signal for enhanced damage clearance. To test this idea, Selby and Sancar (*55*) set up a model system *in vitro* in which the *E. coli* RNA polymerase is blocked at CPDs on a defined template. When they added the purified UvrABC excinuclease, the results indicated that incision was inhibited; however, when they used a crude *E. coli* cell extract, nucleotide-excision repair was enhanced on transcriptionally active DNA (*55*).

Implications of Strand-Specific Repair for Mutagenesis

The existence of preferential DNA repair in expressed genes leads quite directly to the prediction of reduced mutagenesis in these loci in comparison to silent genes. Since the selective repair appears to operate only on the transcribed DNA strand, however, the mutagenic impact should be reduced by only severalfold, and the mutants that do occur can be expected to result from unrepaired lesions in the nontranscribed strand. These predictions have been fulfilled in analysis of UV mutagenesis in the *lac*I gene of *E. coli* (*56*) and in the *hprt* gene of hamster cells (*57*). A strand bias has also been shown for mutations induced by benzo[*a*]pyrene-diol epoxide in human diploid fibroblasts, providing anticipatory evidence that adducts with this diol epoxide are subject to strand-specific repair (*58*). Curiously, a preference for mutations in the non-transcribed strand of *dhfr* in CHO cells treated with *N*-acetoxy-2-acetylaminofluorene has been reported, although, as noted earlier, these cells do not appear to exhibit strand-specific repair of these adducts (*59*). In more recent studies, Carothers and co-workers (*60*) documented strand-specific mutagenesis in the *dhfr* gene induced by ($\pm$)-3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene.

Indirect evidence that the phenomenon of strand-specific repair does not apply to RNA polymerase III-transcribed genes comes from an analysis of Armstrong and Kunz (*61*), in which more UV-induced mutations were found in the transcribed strand in the SUP4-o tRNA gene in yeast. Also, the poor repair of CPDs and psoralen monoadducts in ribosomal genes suggest that RNA polymerase I-transcribed genes do not participate in transcription-coupled repair (*62,63*; F. Christians and P. C. Hanawalt, unpublished observations).

Possible Mechanisms and Future Directions

The accumulated results demonstrate that genomic DNA is not repaired uniformly well in all parts of the genome following chemical and physical insult. Furthermore, the observed heterogeneity of lesion clearance is linked to the active nature of specific loci. A plausible model can be developed on the basis of the collected data to describe preferential DNA repair.

The observation that transcribed strands of genes are better repaired than their nontranscribed counterparts suggests a first level of repair based on enhanced removal of lesions from active genes. Whether specific repair machinery exists for transcription-coupled repair or whether normal nucleotide-excision repair is targeted to this region is not yet clear. Specific proteins might exist that enhance the ability of the repair complex to detect damage in active genes (e.g., genes involved in Cockayne's syndrome).

The results obtained in XP-C cells demonstrating removal of damage only from active domains, imply a level of repair in normal cells that maintains the unexpressed chromatin. At that level, removal of damage may be dependent upon transcription only in the sense that active domains are more accessible to the repair enzymes. Transcription-coupled repair would be a subset of the sequences included at this level.

Regardless of the specifics of the mechanism involved in governing preferential DNA repair, the impact of its existence on biomonitoring and risk assessment is vast. The validity of extrapolating toxicity and carcinogenicity data obtained from nonhuman species has frequently been called into question. Discovery of nonrandom damage and repair adds an additional consideration to interpretation of these results. It may not be sufficient or, in some cases, correct to evaluate the risks of exposure to chemical and physical agents on the basis of total DNA damage and repair; better correlations for the resultant mutagenic, carcinogenic, and toxic consequences may be obtained by investigating adduct clearance from discrete genetic loci.

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